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CELL BIOCHEMISTRY AND FUNCTION
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Effects of polyamines on DNA synthesis using various subcellular DNA polymerases extracted from normal rat liver, tumour-bearing rat liver, and tumour cells

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The effects of polyamines on DNA synthesis *in vitro* using various subcellular DNA polymerase fractions from normal and tumour-bearing rat livers, and tumour cells were investigated. When nuclear and mitochondrial DNA polymerase fractions were used, DNA synthesis on activated DNA was increased 3.5–8-fold by the addition of 20 mM putrescine or cadaverine. However, DNA synthesis was not stimulated by the addition of spermidine or spermine at any concentration tested. In contrast, DNA synthesis using the cytoplasmic DNA polymerase fraction was not stimulated at various concentrations of any of the four polyamines tested. The stimulatory effects of putrescine and cadaverine were absent when nuclear fractions from tumour-bearing rat liver or from tumour cells were used. In addition, *in vitro* DNA synthesis was not stimulated by 20 mM putrescine or cadaverine when nuclear extracts from the livers of rats administered putrescine subcutaneously were used. The specific activities of DNA polymerases extracted from tumour cells and tumour-bearing rat liver were already fully stimulated. These results suggest that DNA polymerases in tumour cells and tumour-bearing liver cells are stimulated by trapped putrescine produced in tumour cells and are thus no longer activated by exogenous putrescine.

KEY WORDS — putrescine; DNA polymerase; stimulation of DNA polymerase; subcellular distribution; stimulation factor; tumour; host liver; tumour detection

INTRODUCTION

It is known that the biosynthesis and accumulation of polyamines are increased in embryos,[1] regenerating liver,[2,3] dividing cultured cells[4] and neoplasmic cells.[5–7] Besides the relationship between the accumulation of polyamines and cell proliferation, the stimulation of DNA synthesis,[7–12] RNA synthesis,[13,14] and protein synthesis,[15,16] by polyamines has also been reported.

With regard to ϕX174 DNA replication, it has been reported that DNA polymerase III star extracted from *Eschericia coli* requires spermidine as a factor for DNA synthesis *in vitro* on ϕX 174 closed circular DNA and an RNA-priming fragment.[17] In mammalian cells, the activity of ornithine decarboxylase, which catalyzes putrescine biosynthesis, increases prior to DNA synthesis. The genes for c-*jun*, c-*fos*, and c-*myc* are expressed in early G1 phase, followed by ornithine decarboxylase and c-H-*ras* expression as the cells move into S phase. Finally, *cdc* kinases, RB protein, thymidine kinase, and histone H3 are induced or activated.[18] In regenerating rat liver, the highest activity of ornithine decarboxylase is found at 16 h and the highest level of DNA synthesis at 26 h after partial hepatectomy.[19] Thus, polyamines are related to DNA synthesis in eukaryotes as well as prokaryotes. Another effect of polyamines on DNA synthesis has been discussed.[8,20] At low concentrations of Mg^{2+}, the stimulation of DNA polymerase activity by the addition of spermidine has been observed. However, the spermidine synthase and spermine synthase activity peaks[21] are later than the first DNA synthesis peak in regenerating liver after partial hepatectomy.

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Therefore, the mechanism of the stimulatory effect by polyamines on cell proliferation in mammals requires investigation.

With the development of methods for measuring polyamine levels, it has been found that the accumulation of putrescine is especially concerned with cell growth.[12,22,23] However, the mode of action of putrescine in DNA synthesis remains unclear.

Here, we will show that DNA synthesis *in vitro* with nuclear or mitochondrial DNA polymerase from normal rat liver is stimulated by putrescine and cadaverine while DNA synthesis with cytoplasmic DNA polymerase is not stimulated. Furthermore, we will also report on differences in the response of DNA synthesis with nuclear DNA polymerases extracted from normal rat liver, host liver of tumour-bearing rats, and tumour tissue itself.

MATERIALS AND METHODS

Chemicals and tissues

[^{3}H]Deoxythymidine 5′-triphosphate was purchased from Dupont/New England Nuclear, Boston, MA, USA. Four unlabelled deoxynucleoside 5′-triphosphates were from Boeringer Mannheim-Yamanouchi Co., Tokyo, Japan. Calf thymus DNA type 1 was from Sigma Chemical Co., St. Louis, MO, USA. Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, and cadaverine dihydrochloride were from Nakarai Chemicals Co., Kyoto, Japan. BCA Protein Assay Reagent was from Pierce Chemical Co., IL, USA.

Regenerating livers were obtained from male Donryu-strain rats 48 h after partial hepatectomy according to the method of Higgins and Anderson.[24] Yoshida ascites hepatoma (AH-130) and Rhodamine sarcoma (Rho-S) cells also from male Donryu strain rats were generously provided by Dr T. Ono (Cancer Institute, Tokyo, Japan).

Inoculation to obtain solid AH-130 tissue was performed by the following procedures. The ascites fluid was collected from an AH-130 bearing rat into centrifuge tubes and centrifuged at 500 g for 5 min. The supernatant was removed and equal volumes of 0.85% NaCl solution were added. The NaCl solution and precipitate were gently mixed, and the suspension was centrifuged again at 700 g for 5 min. This washing was repeated until the removal of contaminating erythrocytes was complete. Finally, the suspension was centrifuged at 1000 g for 10 min. The resulting ascites cells were suspended in equal volumes of the NaCl solution. The maintenance of AH-130 cells was ordinarily performed by inoculating 1 ml of the final suspension intraperitoneally into rats. To obtain solid AH-130 and Rho-S tissues, the same volume of AH-130 cell or chopped Rho-s tissue suspension was inoculated into the dorsal subcutis of Donryu rats. To study the effects of subcutaneously administrated putrescine on rat liver, 0.1 mmole of putrescine-Kg^{-1} body weight was injected. All experimental procedures using laboratory animals were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute of Gerontology.

Preparation of subcellular DNA polymerase fractions

The livers of Donryu strain rats (250–350 g body weight) were used. The livers were homogenized by three strokes of a Teflon homogenizer with 2 volumes of 0.05 M Tris-HCl buffer, pH 8.0, containing 0.34 M sucrose, 25 mM KCl, and 5 mM $MgCl_2$ (buffer A). The homogenate was centrifuged at 1000 g for 10 min. The supernatant fraction was used for the preparation of cytoplasmic and mitochondrial DNA polymerase fractions. The precipitate was resuspended in 9 volumes of 2.2 M sucrose containing 3 mM $MgCl_2$ and centrifuged at 40 000 g for 60 min. The purified nuclear pellet was resuspended in an equal volume of 0.1 M Tris-HCl buffer, pH 8.0, containing 1 mM 2-mercaptoethanol and 1% Triton x-100 (buffer B). This suspension was used as the nuclear fraction. For the extraction of DNA polymerase, the suspension was sonicated for 2 min at setting 3 in a Sonifier Cell Disrupter (model 185, Branson Sonic Power Co., New York, NY, USA). The sonicate was centrifuged at 100 000 g for 60 min. The supernatant fraction was used as the nuclear DNA polymerase fraction. To obtain the cytoplasmic and mitochondrial DNA polymerase fractions, the supernatant from the 1000 g centrifugation described above was centrifuged at 10 000 g for 20 min and the supernatant was further centrifuged at 100 000 g for 120 min. The last supernatant fraction was used as the cytoplasmic DNA polymerase fraction. The precipitate from the 10 000 g centrifugation was resuspended in an equal volume of buffer A and the suspension was recentrifuged at 10 000 g for 20 min. The precipitate was resuspended in an equal volume of buffer B and the extraction of mitochondrial DNA polymerase was carried out by the same procedure described for the nuclear enzyme extraction. The supernatant from the 100 000 g centrifugation was used as the mitochondrial DNA polymerase fraction.

Assays of DNA polymerase activitiy

DNA polymerase activity was measured by the procedure of Taguchi and Ono.[25] The standard reaction mixture contained 0.5 μCi per 0.125 nmoles of [^{3}H] dTTP, 37.5 nmoles each of dATP, dCTP, and dGTP, 2 μmoles of $MgCl_2$, 3 μmoles of dithiothreitol, 5 μmoles of Tris-HCl buffer, pH 8.3, polyamines at the various concentrations indicated on the abscissas of the figures, 20 μg of activated calf thymus DNA, and 0.05 ml of enzyme fraction in a final volume of 250 μl. The reaction mixture was incubated for 30 min at 37°C. At the end of incubation, a 50 μl aliquot from each reaction mixture was applied to a Whatman 3 MM paper disc, 2.4 cm in diameter, previously soaked in 0.1 M $Na_4P_2O_7$ and dried. These discs were then quickly immersed in a large volume of cold 5% trichloroacetic acid (TCA) solution. After 15 min, the TCA solution was discarded by decantation. This washing was repeated three times and the discs were finally placed in 95% ethanol. The discs were dried and put in counting vials containing 10 ml of toluene scintillator composed of 5 g of 2,5-diphenyloxazole (PPO) and 0.1 g of 1,4-bis-2(5-phenyloxazole)-benzene (POPOP) per litre of toluene. The radioactivity was counted in an Aloka LSC-650 liquid scintillation spectrometer (Aloka Co., Ltd., Tokyo, Japan). The amount of DNA synthesis *in vitro* was expressed as pmoles of [^{3}H]dTMP incorporated into DNA, with 1 pmole equivalent to 227 c.p.m.

Measurement of protein content

The protein contents of the enzyme extracts were measured by a modification of the Lowry method using a BCA Protein Assay Reagent with bovine serum albumin as the standard.

RESULTS

DNA synthesis using isolated nuclei from rat liver was not affected by the addition of spermidine or spermine at concentrations below 4 mM, as shown in Figure 1. On the other hand, the amount of DNA synthesis was increased about three-fold by the addition of 20 mM putrescine and about five-fold by the addition of 100 mM cadaverine. To investigate the cause of this stimulation, the effects of polyamines on DNA synthesis with nuclear extracts containing DNA polymerase were examined. As shown in Figure 2, DNA synthesis using this nuclear extract was stimulated about 3.5-fold by the presence of 20 mM putrescine or cadaverine in the reaction mixture. However, DNA synthesis

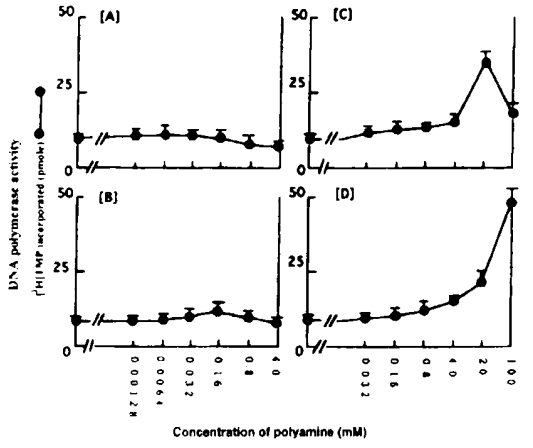

Figure 1. Effects of spermidine (A), spermine (B), putrescine (C) and cadaverine (D) on DNA synthesis by isolated nuclei from rat livers. Rat liver nuclei were isolated by the procedures described in Materials and Methods. For the measurement of DNA synthesis, the reaction mixture contained 0.05 ml of nuclear suspension instead of the DNA polymerase fraction and activated DNA in the standard reaction mixture was used. The amount of synthesized DNA (●——●) was measured as described in Materials and Methods. Values represent means ± SD ($n = 3$) of amounts of synthesized DNA

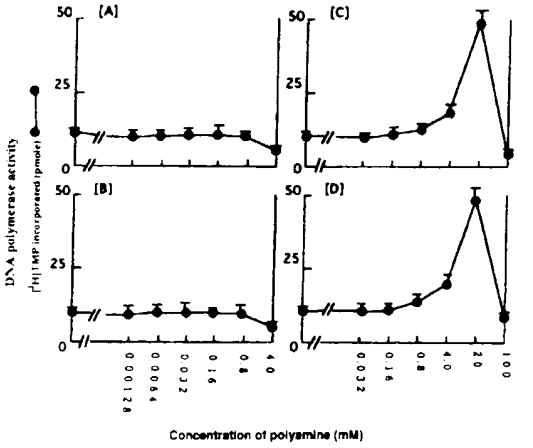

Figure 2. Effects of spermidine (A), spermine (B), putrescine (C) and cadaverine (D) on DNA synthesis by the nuclear DNA polymerase fraction. The nuclear DNA polymerase fraction was extracted from rat liver as described in Materials and Methods. The DNA polymerase activity (●——●) was measured as described in Materials and Methods. Values represent means ± SD ($n = 3$) of DNA polymerase activity

was strongly inhibited by 100 mM putrescine or cadaverine. Further, the effects of spermidine and spermine on DNA synthesis using nucleoplasm were investigated. At spermidine or spermine concentrations lower than 0.8 mM, the amount of DNA synthesis was not changed, but synthesis was decreased by 4 mM spermidine or spermine.

It has been reported that in addition to nucleoplasm, DNA polymerase is also distributed in such subcellular fractions as the cytoplasmic and mitochondrial extracts.[26,27] We were interested in the effects of polyamines on DNA synthesis by DNA polymerases in subcellular fractions other than nucleoplasm. In DNA synthesis with the cytoplasmic DNA polymerase fraction, no stimulatory effects were observed using any of the four polyamines at various concentrations. However, inhibitory effects were found at concentrations of spermidine or spermine higher than 0.8 mM and of putrescine or cadaverine higher than 20 mM (data not shown).

In the following experiments, the effects of polyamines on DNA synthesis with the mitochondrial DNA polymerase fraction were examined. As shown in Figure 3, the results were similar to those obtained for DNA synthesis using the nuclear DNA polymerase fraction.

The incorporation of [^{3}H]dTMP was increased about eight-fold or more by the addition of 20 mM putrescine or cadaverine. These stimulatory effects were greater than those found for DNA synthesis using the nucleoplasmic fraction. First, we hypothesized that the effect of putrescine on DNA synthesis is due to direct action on DNA polymerase. It has been accepted that DNA polymerases α, β, δ, and ε are present in nuclei and that DNA polymerase γ is present in mitochondria.[28–30] However, DNA polymerase α is often found in the cytoplasm as a result of leakage from the nuclei during subcellular fractionation.[31,32] Therefore, the effects of putrescine on the three purified DNA polymerases were examined. DNA synthesis by the partially purified DNA polymerase α, β, or γ was not stimulated by any of the four polyamines at any concentration (data not shown).

In proliferative cells, it has been reported that ornithine decarboxylase (ODC) activity increases prior to DNA synthesis.[19,33] These reports suggested that putrescine is necessary for DNA replication. Therefore, the effects of polyamines on DNA synthesis using nuclear extracts from tumour cells and regenerating liver cells were also investigated. Actually, DNA synthesis using nuclear extracts from AH-130 cells was not stimulated by putrescine at various concentrations (Figure 4). Similar results were obtained in experiments of DNA synthesis with nuclear extracts from Rho-S cells, from regenerating livers 24 and 48 h after partial hepatectomy, and from fetal liver (data not shown). Further, DNA synthesis with nuclear extracts from host livers of AH-130- or

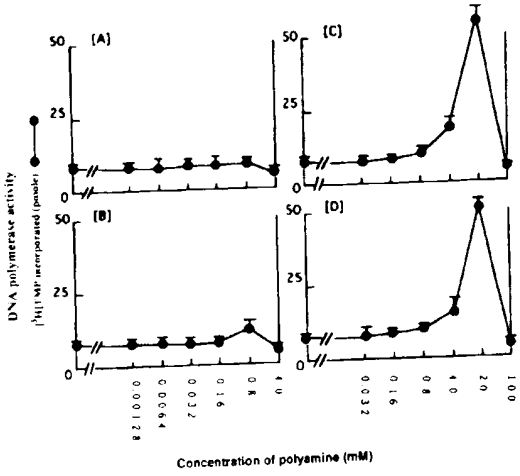

Figure 3. Effects of spermidine (A), spermine (B), putrescine (C) and cadaverine (D) on DNA synthesis by the mitochondrial DNA polymerase fraction. The mitochondrial DNA polymerase fraction was extracted from rat liver as described in Materials and Methods. The DNA polymerase activity (●—●) was measured as described in Materials and Methods. Values represent means ± SD (n = 3) of DNA polymerase activity

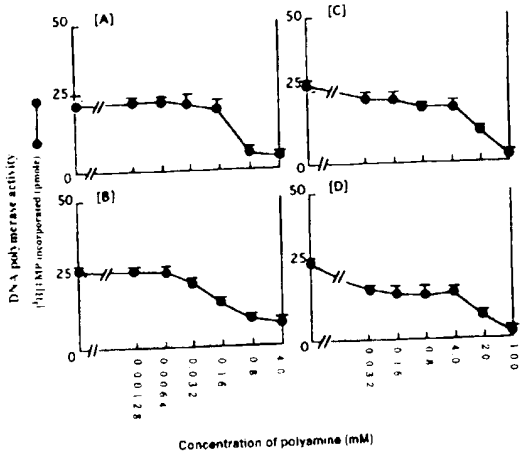

Figure 4. Effects of spermidine (A), spermine (B), putrescine (C) and cadaverine (D) on DNA synthesis by the nuclear DNA polymerase fraction from AH-130 cells. The nuclear DNA polymerase fraction was extracted from rat liver as described in Materials and Methods. The DNA polymerase activity (●—●) was measured as described in Materials and Methods. Values represent means ± SD (n = 3) of the DNA polymerase activity

Rho-S-bearing rats was also not stimulated (data not shown). In addition to these results, no stimulatory effects of DNA synthesis were observed using any mitochondrial extract from the above proliferating cells and from host livers of tumour-bearing rats (data not shown).

We were further interested in the lack of a stimulatory effect on DNA synthesis by nuclear and mitochondrial extracts caused by additional putrescine. This may be the result of putrescine biosynthesis in proliferative cells and tissues. To clarify the mechanism for the lack of stimulatory effects on tumour cells and host liver, we measured the changes in AH-130 tumour weight after inoculation into the backs of rats, the relative specific activity of DNA polymerase in tumour cell extracts, and the effect of 20 mM putrescine on DNA synthesis *in vitro* caused by nuclear extracts from tumour cells. Although AH-130 cell growth was not observed until 2 days after inoculation, the tumour weight increased sigmoidally up until 10 days after that (Figure 5). The relative specific activities of DNA polymerase in nuclear extracts from AH-130 cells were elevated about six-fold over control levels 2 to 4 days after inoculation and remained high beyond 4 days (Figure 5). However, the stimulation of *in vitro* DNA synthesis by 20 mM putrescine was decreased 2 to 4 days after inoculation (Figure 6). As described above, *in vitro* DNA synthesis with nuclear extracts from host livers of AH-130-and Rho-S-bearing rats was not stimulated by the addition of 20 mM putrescine (data not shown). This lack of stimulation may be due to putrescine biosynthesis in rapidly growing tumour cells. If this hypothesis is correct, the lack of stimulation of *in vitro* DNA synthesis by 20 mM putrescine induced by nuclear extracts from the livers of rats bearing subcutaneously injected putrescine would be observed. We prepared nuclear extracts from the livers of rats 0, 1, 2, 3, and 4 days after receiving a subcutaneous injection of putrescine. The incorporation of [^{3}H]dTMP during *in vitro* DNA synthesis induced by extracts of livers immediately after putrescine injection was increased three-fold by the addition of 20 mM putrescine into the reaction mixture. However, no such stimulation was observed using extracts prepared from livers 1 to 4 days after subctaneous putresine injection (Figure 7).

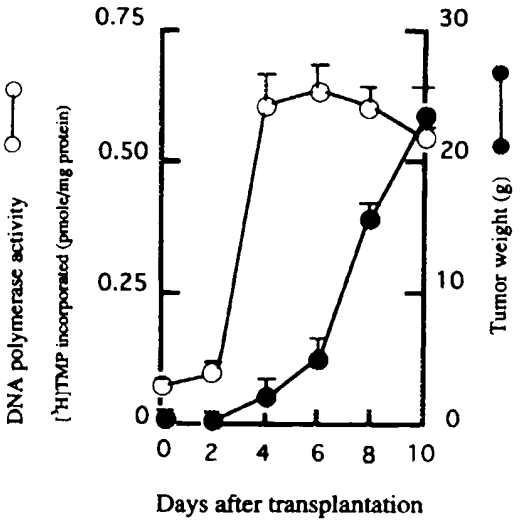

Figure 5. Change of tumour weight (g) (●——●) and specific activity of DNA polymerase (○——○) in host liver nuclei. AH-130 cells packed by 1000 g centrifugation were suspended in equal volumes 0.85% NaCl solution. Of the cell suspension 1 ml was inoculated subcutaneously into the backs of Donryu rats (approx. 200 g body weight). The tumour weights were measured 0, 2, 4, 6, 8, and 10 days after inoculation and the host livers were removed. The nuclear DNA polymerase fractions were extracted from host livers as described in Materials and Methods. The specific DNA polymerase activity was measured as described in Materials and Methods. Values represent means + SD ($n = 5$) of the tumour weights or the specific DNA polymerase activity

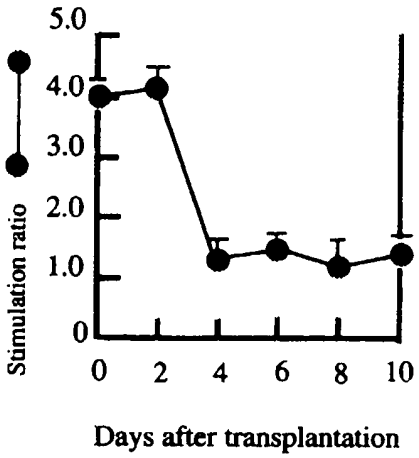

Figure 6. Effects of putrescine on DNA synthesis by nuclear extracts from tumour-bearing host livers. Nuclear extracts were prepared from host livers 0, 2, 4, 6, 8, and 10 days after inoculation with AH-130 cells, and the amount of DNA synthesis by each extract was measured with and without 20 mM putrescine. Stimulation ratios of DNA synthesis by putrescine were plotted (●——●). Values represent means ± SD ($n = 5$) of stimulation ratios of DNA synthesis

DISCUSSION

A stimulation of DNA synthesis by putrescine or cadaverine using nuclear and mitochondrial preparations

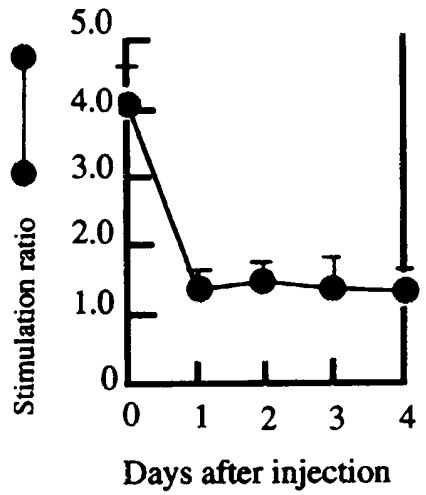

Figure 7. Effects of putrescine on DNA synthesis by nuclear extracts from the livers of rats injected with putrescine. Nuclear extracts from livers 0, 1, 2, 3, and 4 days after putrescine injection were prepared, and the amount of DNA synthesis by each extract was measured with and without 20 mM putrescine. Stimulation ratios of DNA synthesis by putrescine were plotted (●——●). Values represent means ± SD ($n = 5$) of the amount of DNA synthesis or stimulation ratios of DNA synthesis

was observed. The concentration difference for cadaverine effects between nuclei and nucleoplasm may be due to the low rate of penetration into the nuclei. It is accepted that putrescine but not cadaverine is synthesized in mammalian cells. Therefore, the data suggest that DNA synthesis is actually stimulated by putrescine in the nuclei and mitochondria of rat liver. On the other hand, no stimulation of DNA synthesis using cytoplasmic extracts was found when putrescine, cadaverine, spermidine, or spermine was added (data not shown). We have been interested in the different stimulatory effects of putrescine and cadaverine on DNA synthesis using three different crude DNA polymerase fractions. DNA synthesis by the partially purified DNA polymerase α, β, or γ was not stimulated by putrescine. This suggests that the stimulation of *in vitro* DNA synthesis by putrescine is not a direct effect on DNA polymerase itself. From these results, we hypothesize the presence of a certain factor in the nuclei and mitochondria, and that separation of this factor from DNA polymerase would produce a loss of the stimulatory effect on DNA synthesis *in vitro* by putrescine.

The disappearance of the stimulatory effect of exogenously added putrescine on DNA synthesis was also observed when nuclear extracts from proliferating cells such as tumour cells, fetal liver cells, or regenerating liver cells were used. Therefore, we hypothesize that the machinery for DNA synthesis is stimulated by putrescine synthesized *in vivo* and is not further stimulated by exogenous putrescine. It has been reported that the putrescine concentration of normal rat liver is 60 nmoles g^{-1} wet weight.[3] From calculations using this value and our data, endogeneous putrescine concentrations in the reaction mixture for DNA synthesis using extracts of nuclear, mitochondria and cytoplasm are about 0.24, 1.92 and 0.77 mM, respectively, if putrescine distribution is uniform in the cell organelles. Putrescine biosynthesis *in vivo* begins prior to cell proliferation.[19,28] The putrescine concentrations of regenerating rat liver, tumour cells, and tumour-bearing rat liver are increased by entrance to the proliferation state of the cells.[3] The time courses of the rapid growing phase of AH-130 cells and the elevated relative specific activity of nuclear DNA polymerase in tumour cells after subctaneous inoculation are similar. During this period, DNA synthesis using nuclear extracts from AH-130 cells was not increased by the addition of exogenous putrescine. These results support our hypothesis.

On the other hand, DNA synthesis using nuclear and mitochondrial extracts from tumour-bearing liver was not stimulated by exogenous putrescine. In this case, putrescine synthesized in tumour cells may be brought to the liver by the bloodstream. The transport of putrescine by the bloodstream is supported by the experiments in which putrescine was injected subcutaneously. Despite the administration of putrescine at a dose of 0.1 mmole of putrescine Kg^{-1} body weight, no stimulation of DNA synthesis by exogenous putrescine was found, even though the stimulation of DNA synthesis *in vitro* requires 20 mM putrescine. However, the physiological concentration of putrescine in cells is not high. Therefore, in proliferating cells and host liver cells, a system for trapping putrescine to stimulate DNA polymerase activity may be present, and this system contains a factor that allows the trapping of putrescine *in vivo*. DNA polymerase stimulated *in vivo* by putrescine is not further stimulated by exogenous putrescine. As described above, putrescine concentration in the nuclear extract from normal rat liver is 0.24 mM, and the value in that from regenerating liver is elevated to about 1.32 mM. Therefore, a trapping system is necessary to explain the putrescine concentration effects. A factor related to the stimulation of DNA synthesis by putrescine or cadaverine may be essential for the putrescine trapping system. To clarify this, further studies are needed.

It has been reported that DNA synthesis *in vitro* is stimulated by the addition of polyamines at low Mg^{2+} concentrations.[8,20,34] Certainly, DNA synthesis by nuclear and mitochondrial DNA polymerase fractions is stimulated by the addition of 20 mM putrescine or cadaverine at Mg^{2+} concentrations of 1.0, 2.0, or 4.0 mM (data not shown). However, the amount of DNA synthesis by nuclear DNA polymerase fractions at any concentration below 8 mM Mg^{2+} and 20 mM putrescine never exceeds that at the optimal $MgCl_2$ concentration (8 mM) in the absence of putrescine. In our experiments, 8 mM $MgCl_2$ was added to each DNA synthesis assay. This Mg^{2+} concentration is optimum or nearly optimum for assays using any fraction as the source of DNA polymerase. Under these conditions, putrescine further elevates the rate of DNA synthesis, and this stimulation requires a specific factor. Therefore, our observation is different from the stimulation by polyamines seen at low Mg^{2+} concentrations.

Some reports[8–12,35,36] have shown DNA synthesis to be stimulated by polyamines. In DNA synthesis by nuclei from *Physarum polycephalum*, the incorporation of [^{3}H]dATP is increased by spermine; and DNA synthesis by DNA polymerase III star from *Escherichia coli* requires spermidine for complete activity.[10,11] On the other hand, in our experiments, the polyamines that stimulated DNA synthesis were putrescine and cadaverine. As putrescine is synthesized in mammalian cells, DNA synthesis is actually stimulated by putrescine in the nuclei and mitochondria of rat liver. These differences in polyamines may be due to differences in the species specificities of DNA polymerase preparations or to mechanical steps related to polyamine action. A correlation between the accumulation of putrescine and the stimulation of cell proliferation has been reported several times.[1,3,4,34] The stimulation of cell proliferation by putrescine may be due to an increase in DNA synthesis caused by putrescine as indicated by our findings.

It is very important to clarify whether DNA synthesis by blood cell lysates from rats with and without tumours is stimulated by exogenous putrescine or not. If stimulation is found in control rats and not in tumour-bearing rats, then this may be useful for the diagnosis of tumours in patients other than pregnant women and babies.

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